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FACILITATED TRANSPORT OF DI- AND TRINITROPHENOLATE IONS ACROSS LIPID MEMBRANES BY VALINOMYCIN AND NONACTIN

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SUMMARY

The conductance of black lipid membranes in the presence of 2,4,6-trinitrophenol (or 2,4-dinitrophenol) is considerably enhanced, if the cation carriers valinomycin, enniatin B or nonactin are added. The effect is, however, largely independent of the cation concentration and is identical for the cations Li^+, Na^+ and Ba^{2+}. This finding, as well as the sign and magnitude of the diffusion potential in the presence of a gradient of picrate are consistent with the assumption that the transport of picrate anions is facilitated by the above-mentioned macrocyclic compounds, but that cations are not directly involved. A model is suggested which, based on the generation of mobile defect structures by the incorporation of large molecules, allows one to explain facilitated transport without the assumption of stable chemical bonds between a carrier and its transported substrate.

If K^+ is present in the aqueous phase, the conductance is largely determined by the permeation of the cation complexes of valinomycin and nonactin. The conductance is, however, increased by adsorption of picrate anions to the membrane surface. The negative surface potential generated by the adsorption layer seems to be responsible for the saturation of the conductance at high picrate concentrations in the absence of valinomycin and nonactin.

INTRODUCTION

Thin lipid membranes are widely used at present for the study of the principles of ion transport through thin hydrophobic barriers, such as a biological membrane. The great advantage of this model system resides in its rather well defined structure and especially in the applicability of a wide spectrum of electrical and optical methods. This has become clearly apparent throughout the study of the effect of certain antibiotics and of some uncouplers of oxidative phosphorylation, both of which strongly influence the permeability properties of natural and artificial membranes. Molecules like valinomycin or dinitrophenol have been shown to act as carriers for K^+ and H^+, respectively, while gramicidin or alamethicin are assumed to form aqueous pores through membranes. These findings, extensively reviewed in recent years [1–5],

form the mechanistic bases for the use of ionophores in cell biology. Their application allows one to generate or to abolish electrical diffusion potentials across biomembranes and in this way to contribute to the study of the potential dependence of biologically important membrane phenomena, such as oxidative phosphorylation. Throughout these studies, more than one ionophore is often simultaneously incorporated into a given membrane in order to create an increased permeability for several cations or for cations and anions. Thereby, an independent action for the single ionophores is tacitly assumed. This assumption, which may considerably influence the interpretation of cell biological experiments, can be submitted to a critical test in artificial lipid membranes. Such a test will also help to mark the range where ionophores like nonactin can be used to estimate changes in the electrostatic potential at membrane interfaces, a procedure proposed with considerable success by McLaughlin [9].

A few attempts to study the interaction of different transport systems in lipid membranes have been reported. Kuo and Bruner [6] found that the uncoupler 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole can block valinomycin-induced potassium conductance in bilayer membranes. The effect was interpreted as a competition of both ionophores for a limited number of adsorption sites available at the membrane surface. Similar observations and conclusions were reported by Demin et al. [7] for valinomycin and high concentrations of the lipophilic anion tetraphenylboron. One of the most outstanding properties of the ion carrier valinomycin is its large preference for potassium over sodium. The conductance increase of lipid membranes induced by this ionophore is 10^3–10^4 times larger in the presence of potassium compared to sodium, and mirrors the selectivity of the association constant of complex formation in methanol [3, 5]. Tosteson [8], however, reported that the selectivity of valinomycin is almost completely abolished, if certain nitrophenolates (trinitrocresolate and trinitrophenolate are most potent in this respect) are added to the aqueous phases on both sides of the membrane. Then, the conductance in the presence of sodium has a similar value as in the presence of potassium. He suggested that the anions mentioned above might change the distribution of conformational states of the cyclic depsipeptide in such a way that the selectivity of complex formation with the monovalent cations is reduced.

This paper presents further experimental evidence for a synergistic action of valinomycin and nitrophenolates in lipid membranes. It was found that the diffusion of nitrophenolate anions across these membranes is facilitated by valinomycin. This surprising effect is also produced by other compounds of sufficient molecular size such as nonactin or enniatin. A model is suggested which allows one to understand how the movement of small molecules across a bimolecular lipid structure might be influenced by the presence of larger particles. It is not based on specific "carrier-substrate interactions", but considers the lattice defects in the vicinity of large particles. Thus, a new interpretation for the finding of Tosteson [8] is obtained.

MATERIALS AND METHODS

Black lipid membranes were formed from dioleoyllecithin, which was synthesized by K. Janko (University Konstanz). The cell used for membrane formation as well as the technique of steady-state and pre-steady-state conductance measure-

ments have been described previously [10, 11]. When valinomycin was present in the aqueous phases, the aperture in the wall separating the aqueous compartments in most cases had an area of about 0.1 cm^2 in order to minimize the influence of the torus [10]. The equilibration of valinomycin between membrane and water in the presence of picrate (2,4,6-trinitrophenol) was found to depend on the concentration of picrate. In some cases more than 100 min were needed to obtain a constant value of the conductance. All experiments were carried out at room temperature (22 ± 1 °C). Electrolyte solutions were made with analytical grade reagents in double-distilled water. The pH of the unbuffered aqueous solutions was about 6.

In most figures the initial conductance λ_{00} is plotted in order to account for the rather slow relaxation of the current (see Fig. 2). The amplitude of this relaxation normally was very small. In the presence of valinomycin and picrate (in the absence of K^+) the decrease of the current within the first 10 s on application of the voltage was only about 15 %. Therefore, the use of a Keithly electrometer (Model 610 C) with a rise time of less than 1 s, was considered sufficient to measure the "initial current", defined as the current at the end of the charging period of the membrane capacity. In other cases, the voltage jump method (pulse generator in combination with an oscilloscope) was applied to follow the time course of the current [11, 12]. At the measurements of the open circuit potentials the membranes were formed with identical aqueous solutions in both compartments. Then, the solution of one compartment was exchanged under stirring against a solution of different ionic concentrations (as mentioned in the legends) and the electrical potential was measured with a Keithly electrometer.

RESULTS

(*A*) *The potassium-free system*

The outstanding effect of valinomycin on the cation permeability of membranes is largely confined to rubidium, potassium and cesium, whereas sodium, lithium or divalent ions are scarcely transported. We will denote the first group of monovalent cations as the "potassium group" and first study the synergistic action of valinomycin and 2,4,6-trinitrophenol in the absence of this group of cations. The conductance of black films in the presence of LiCl in the aqueous phases is below 10^{-7} $(\Omega \cdot cm^2)^{-1}$ and is hardly influenced by the presence of valinomycin or nonactin. If in the absence of valinomycin or nonactin picrate is added to the aqueous phases, the membrane conductance first shows an increase, but finally saturates at concentrations above 10^{-3} M (Fig. 1). The pK of this compound is about 0.4 [13]. Therefore, at pH 6 virtually all molecules are present in the anionic form and the conductance increase is due to a permeation of the picrate anions. This is consistent with the observation that the conductance is largely independent of the pH. Only at low pH was an increase reported, which seems to indicate a voltage-dependent translocation of protons [14, 15]. The origin of the saturation found at high picrate concentrations (Fig. 1) will be discussed in detail later in this paper. Evidence will be presented that picrate anions are adsorbed to the membrane interface and produce some kind of "selfhindrance" via the negative surface potential generated by their charge. If valinomycin and picrate are present simultaneoulsy in the aqueous phases a very distinct conductance increase compared to the pure picrate system is observed, which

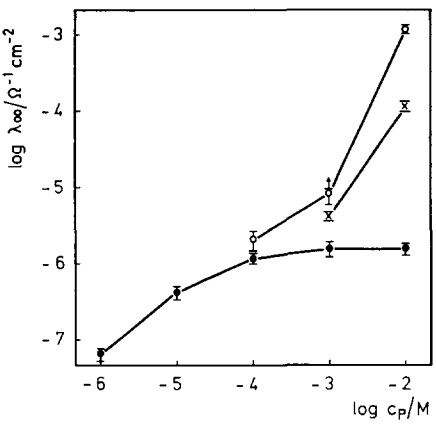

Fig. 1. Initial conductance λ_{00} as a function of picrate concentration without (●) and with 10^{-7} M valinomycin (○) or 10^{-6} M nonactin (×) in the aqueous solutions. The pH was adjusted to about 6 using LiOH. The total ionic strength was held constant at 11 mM with LiCl. The data represent mean values of five membranes, the horizontal bars show twice the standard error. The arrow at 10^{-3} M picrate in the presence of valinomycin indicates that the equilibrium between membrane and water was not reached (see text).

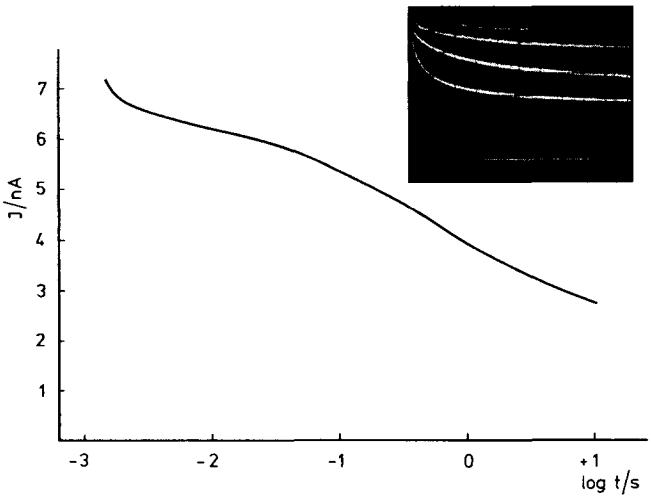

Fig. 2. Time course of the current following a voltage jump of 16 mV. The aqueous solutions contained 10^{-2} M picrate (pH 6 with LiOH), 10^{-3} M LiCl and 10^{-6} M nonactin. The current was corrected for a small voltage drop in the aqueous phases (resistance without membrane $2.2 \cdot 10^4\ \Omega$) and for the external resistor ($R_A = 10^5\ \Omega$). The membrane area was $5 \cdot 10^{-3}$ cm^2. The inset shows the original oscilloscope picture with a sensitivity of 0.1 mV/cm and different time scales (1 ms/cm; 10 ms/cm; 0.1 s/cm; 1 s/cm). The lowest trace corresponds to the base line.

amounts to roughly three orders of magnitude at 10^{-2} M picrate and 10^{-7} M valinomycin. The time necessary to attain a constant conductance is very prolonged, if both species are present. While in the presence of picrate alone the conductance reaches its final value about 5–10 min after the membrane has become fully black, this time period lasts considerably longer if valinomycin is present in addition. At 10^{-3} M picrate a continuous small increase in the conductance was found even after 100 min (see arrow). A similar but less pronounced effect on the conductance was found if valinomycin was replaced by another potassium carrier nonactin. On the ordinate of Fig. 1, as well as on that of some of the following figures, the initial conductance λ_{00} in the ohmic region of the membrane is plotted. This initial conductance is defined as follows. With picrate or with valinomycin (nonactin) alone no current relaxation could be detected within a time range of milliseconds to seconds. In the presence of both compounds, however, the current, following a voltage jump shows a permanent decrease extending over at least four orders of magnitude in time. Fig. 2 shows an example. The current spike stems from the loading of the membrane capacity, which, with the given experimental conditions, is completed after about 2 ms. The initial conductance λ_{00} refers to the initial current, which is fairly constant over the first 10 ms. The amplitude of this current relaxation has been found to be considerably larger for the system nonactin/picrate as compared to valinomycin/picrate. According to Fig. 2 the current has decreased to about 40 % of the initial value after 10 s. The same experiment with 10^{-7} M valinomycin instead of nonactin, though showing a similar time course, gave a value of about 85 %.

The combined effect of valinomycin (nonactin) and nitrophenolate anions on the conductance of lipid membranes is also present with 2,4-dinitrophenol. Fig. 3 shows, however, a less pronounced conductance increase induced by the neutral compounds compared to trinitrophenol. Moreover, nonactin is more effective in this system than valinomycin. Again a current relaxation was observed, but only at 10^{-2} M dinitrophenol and in the presence of one of the ionophores. The results of Figs. 1 and 3 cast some doubts upon the applicability of ionophores for the assessment of the surface charge density produced by the adsorption of surface active ions. McLaughlin [9] used the nonactin/K^+ system to estimate the charge density of

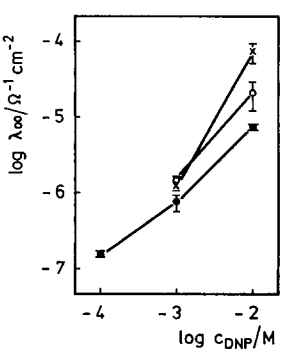

Fig. 3. Conductance λ_{00} as a function of 2,4-dinitrophenol concentration in the absence (●) and presence of 10^{-7} M valinomycin (○) or 10^{-6} M nonactin (×) in the aqueous solutions (containing 0.1 M LiCl). The pH was adjusted to 7 using LiOH. The horizontal bars indicate twice the standard error.

adsorbed dinitrophenol and reported that the almost linear relationship between conductance and dinitrophenol-concentration (see Fig. 3) is converted into a second-order dependence, if the data are corrected for the negative surface potential of the membrane. This procedure is only correct in the absence of a direct interaction of both transport systems. Such an interaction follows from the results presented above. Nevertheless, McLaughlin's interpretation remains correct, since he restricted his measurements to concentrations below 10^{-3} M, where, according to Fig. 3, the combined effect of dinitrophenol and nonactin is small. We will use the same approach for an evaluation of the "true" concentration dependence of the conductance induced by trinitrophenol, later in this paper.

We will now concentrate on the nature of the charge carriers which are responsible for the increase in conductance induced by valinomycin. The synergistic effect depends strongly on the picrate concentration (Fig. 1), which indicates its direct involvement. The same conclusion is obtained for valinomycin (or nonactin). Fig. 4 shows a roughly linear dependence of λ_{00} on their concentration in water at constant picrate and cation concentrations. The reason for the weaker dependence, if valinomycin is added to the membrane-forming solution, is unknown at present. It might be related to the rather complex equilibration process of valinomycin between torus and membrane. Valinomycin and nonactin are known as excellent cation carriers. The question arises, therefore, whether cations are directly involved in the observed phenomena. An involvement of cation complexes would require a change in the complexation properties of valinomycin, which strongly favors K^+ over Li^+. Such a change was postulated by Tosteson [8], who suggested a loss of the cation selectivity of valinomycin induced by picrate. Therefore, the dependence of the conductance on the presence of cations was studied. The minimum amount of cations, which must be added as hydroxide to obtain a pH of about 6, roughly corresponds to the concentration of the acid trinitrophenol. In Fig. 5 the Li^+ concentration was

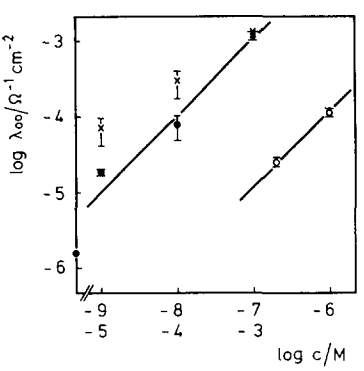

Fig. 4. Initial conductance λ_{00} as a function of valinomycin and nonactin concentration with 10^{-2} M picrate in the aqueous solutions: ●, valinomycin and 10^{-3} M LiCl in water; ○, nonactin and 10^{-3} M LiCl in water; ×, addition of valinomycin to the lipid, 10^{-3} M NaCl in water. The pH was adjusted to 6 using LiOH or NaOH, respectively, so that the total cation concentration was about 11 mM. The data represent mean values of five membranes with the bars indicating the standard error. The upper abscissa corresponds to addition of the carrier molecules to water, while the lower abscissa corresponds to addition to the membrane-forming solution. Full lines were drawn with slope 1.

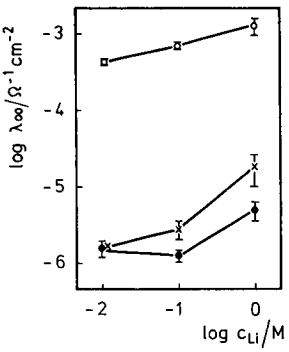

Fig. 5. Initial conductance λ_{00} as a function of ionic strength (11 mM–1 M) adjusted with LiCl (pH 6 with LiOH): ●, 10^{-3} M picrate; ×, 10^{-2} M picrate; ○, 10^{-2} M picrate and 10^{-7} valinomycin in water. The data were obtained using membranes with an area of 0.025 cm^2. Therefore, the conductance values per cm^2 found in the system valinomycin/picrate are smaller than those given in Fig. 1 (membrane area 0.1 cm^2) because of the torus effect [11].

varied between 11 mM and 1 M. It was accompanied by approximately a 3-fold conductance increase in the valinomycin/picrate system. This relatively small effect hardly supports a direct participation of Li^+ as component of a supposed charge carrier complex, since a similar effect was also found in the absence of valinomycin. The small conductance increase found at high ionic strength, seems to be based rather on a reduction of the negative surface potential which is generated by adsorption of picrate anions and which gives rise to the saturation of picrate conductance at high concentrations (see Fig. 1 and next section). This surface potential diminishes the concentration of anions near the membrane interface and concomitantly reduces the concentration of negative charge carriers in the membrane. Evidence for a negative charge carrier also in the case of the valinomycin/picrate system will be presented below. The idea that cations are only indirectly involved obtains further support by the complete absence of any specificity. The conductance at constant ionic strength remains the same if Li^+ is replaced by Na^+ or even by the divalent Ba^{2+} (Table I). The equal conductance values for Na^+ and Li^+ might be understood on the basis of a changed conformation of valinomycin which has lost the ability of discrimination between different monovalent cations. This argument, however, is not applicable to the divalent cation Ba^{2+}, since a replacement of mono- by divalent

TABLE I

DEPENDENCE OF CONDUCTANCE (in $\Omega^{-1} \cdot cm^{-2}$) ON THE KIND OF CATION PRESENT IN WATER, WITH AND WITHOUT VALINOMYCIN

The data were obtained with 10^{-2} M picrate in water. The pH was adjusted to 6 using the hydroxide of the corresponding cation.

	No valinomycin	10^{-7} M valinomycin
10^{-3} M LiCl	$(1.6 \pm 0.2) \cdot 10^{-6}$	$(1.2 \pm 0.14) \cdot 10^{-3}$
10^{-3} M NaCl	$(1.5 \pm 0.3) \cdot 10^{-6}$	$(1.2 \pm 0.16) \cdot 10^{-3}$
$5 \cdot 10^{-4}$ M $BaCl_2$	$(1.7 \pm 0.7) \cdot 10^{-6}$	$(1 \pm 0.7) \cdot 10^{-3}$

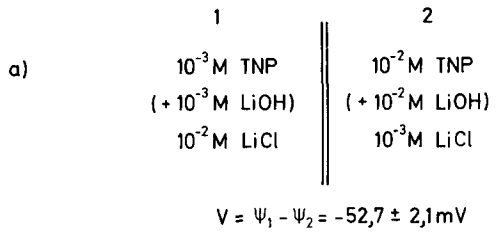

Fig. 6. Open circuit potential in the presence of a 10-fold gradient of trinitrophenol with and without valinomycin (mean values of five membranes with standard error). The ionic strength on both sides was adjusted to 11 mM by adding LiCl. The measurements were performed using calomel electrodes filled with 1 M LiCl.

cations would change the valency of the charge carrier and, as an almost inevitable consequence, would also change the conductance of the membrane. Further experiments, which are not presented in Table I, have shown that the combined effect of valinomycin and picrate is also present, if the pH is adjusted by the Tris cation, i.e. in the complete absence of alkali or alkaline earth cations.

These experiments support a hypothesis which does not directly involve cations, and which is based on the assumption of a facilitation of the transport of picrate anions by the neutral compounds valinomycin and nonactin. This hypothesis was tested by the measurement of open circuit potentials in the presence of a 10-fold gradient of picrate. Fig. 6 shows that the diffusion potential obtained in the absence or presence of valinomycin corresponds almost ideally to the equilibrium potential for a monovalent negative charge carrier (−58.5 mV). This may indicate that in both cases picrate anions are substantially involved. A possible explanation of the effect of valinomycin is the assumption of a "normal" carrier mechanism. This probably represents the simplest way of understanding facilitated transport across membranes and suggests itself in view of the known "carrier activity" of valinomycin for monovalent cations. In this case, however, the facilitated movement of picrate across lipid membranes would require a specific molecular interaction between

TABLE II

CONDUCTANCE IN THE PRESENCE OF DIFFERENT NEUTRAL, MACROCYCLIC COMPOUNDS AND 10^{-2} M PICRATE ($+10^{-3}$ M LiCl) IN THE AQUEOUS SOLUTIONS

The pH was about 6 using LiOH. In the absence of picrate, λ_0 was smaller than $10^{-7}\ \Omega^{-1} \cdot cm^{-2}$ in all cases (membrane area 0.1 cm^2).

	No additive	10^{-7} M valinomycin	10^{-6} M nonactin	10^{-5} M enniatin B
$\lambda_{00}/\Omega^{-1}\ cm^{-2}$	$1.6 \cdot 10^{-6}$	$1.2 \cdot 10^{-3}$	$1.1 \cdot 10^{-4}$	$3 \cdot 10^{-4}$

valinomycin and picrate anions. Though some indications for such an interaction have been reported [8, 16], further mechanisms have been envisaged, which do not make use of specific molecular interactions between valinomycin and nitrophenolates. The reason for the search for alternatives to a carrier mechanism has been the fact that the increase of picrate permeability is not restricted to valinomycin. Table II shows that apart from valinomycin and nonactin, enniatin B is also able to produce a substantial conductance increase in the presence of 10^{-2} M picrate. All three neutral macrocyclic compounds are known as K^+ carriers across membranes [17], but ineffective in the presence of Li^+. The fact that these substances also promote the permeation of picrate anions raises the question whether all of them, though of different chemical structure, should be able to bind these anions. This should be more difficult to explain than their complex formation with cations. Therefore, alternative ideas of facilitated transport have been presented, which do not depend on the existence of chemically stable carrier-ion complexes (see Discussion).

(B) The potassium system

The experiments reported in the last section were performed in the absence of ions which are normally transported by the cation carriers valinomycin and nonactin. They enabled us to study the effect of these ionophores on the membrane permeability of picrate anions without interference of a high cation permeability. We will now proceed to the more complicated case, where potassium was present in the aqueous phases. Here, the conductance assumes a relatively high value in the presence of valinomycin (or nonactin) and K^+ alone (Fig. 7). A further conductance increase, however, is induced by adding picrate. In addition, the presence of these

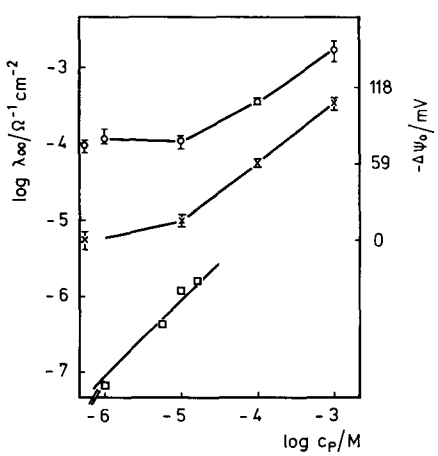

Fig. 7. Conductance induced by valinomycin (nonactin) and potassium as a function of picrate concentration: ○, 10^{-7} M valinomycin, 10^{-2} M KCl; ×, 10^{-6} M nonactin, 10^{-2} M KCl. The pH was adjusted to 6 using LiOH. The membrane area was $2.5 \cdot 10^{-2}$ cm^2. Since the conductance increase is presumably due to the adsorption of picrate to the membrane surface, the change of the electrical potential calculated according Eqn. 3 from the nonactin data is indicated on the right hand ordinate. □, the concentration dependence of λ_0 obtained in the presence of picrate alone (Fig. 1, full circles) was corrected for the surface potential by multiplying the bulk concentrations with the factor $e^{F\Delta\Psi_0/RT}$ (see text). The full line was drawn with slope 1.

anions gives rise to the emergence of a current relaxation already observed with the the potassium-free system (Fig. 2). Therefore, the initial conductance λ_{00} was plotted. In the absence of picrate it is identical to the steady-state conductance λ_0*. The concentration of picrate was varied up to 10^{-3} M. In this concentration range the conductance increase induced by picrate was more than one order of magnitude larger than the corresponding increase in the potassium-free system (compare Fig. 1). On the other hand, the facilitation of picrate transport by valinomycin (or nonactin) has been found to be independent of the kind of cation present in the aqueous solutions. This suggests that an additional effect dominates in the presence of K^+. It has been mentioned already in section A that the saturation of the conductance observed at high picrate concentrations might stem from an electrical selfhindrance due to an adsorption of picrate anions to the membrane-water interface. The negative surface potential generated in this way would increase the cation concentration near the membrane and therefore also the conductance, which is induced by the normal valinomycin/K^+ system. It has been shown previously [9, 10] that, apart from saturation effects at high potassium concentrations, a linear relationship exists between the conductance λ_0 induced by valinomycin/K^+ and the potassium concentration $c_K{}^i$ at the membrane interface:

$$\lambda_0 = B \cdot c_K^i \tag{1}$$

B is a constant, which depends linearly on the carrier concentration. On the other hand, the concentration $c_K{}^i$ is related to the bulk concentration c_K by

$$c_K^i = c_K \cdot e^{-F\Psi_0/RT} \tag{2}$$

with F = Faraday constant, R = gas constant, T = temperature. Ψ_0 is the electrical potential difference between interface and bulk aqueous phase. The change of the electrostatic potential $\Delta\Psi_0$, which is due to the adsorption of picrate anions, influences the conductance induced by valinomycin/K^+. With Eqns. 1 and 2:

$$\lambda_0 = \lambda_0^0 e^{-F\Delta\Psi_0/RT} \tag{3}$$

$\lambda_0{}^0$ is the conductance induced by valinomycin/K^+ in the absence of picrate. Eqn. 3 allows to calculate the potential difference $\Delta\Psi_0$ (right hand ordinate of Fig. 7). The nonactin data have been used, since the macrotetrolides show a larger range of proportionality between λ_0 and c_K than valinomycin [10]. The initial conductance λ_{00} was tacitly treated like the steady-state conductance λ_0. A justification is given later.

If the saturation of the membrane conductance in the presence of picrate alone (Fig. 1) is generated by electrostatic selfhindrance, the "true" concentration dependence of λ_0 may be calculated by applying the same considerations to the anion picrate. Its concentration near the interface is lowered by a factor $e^{F\Delta\Psi_0/RT}$. Therefore, the conductance data of Fig. 1 have to be correlated to the interfacial picrate concentrations instead of the bulk concentrations. Fig. 7 shows that after this correction a roughly linear relationship between conductance and interfacial con-

* The amplitude of the very fast current relaxation previously observed with valinomycin-mediated K^+ transport [11, 12] is negligible with the experimental conditions of Fig. 7. Besides, the corresponding time constant would be in the μs region.

centration is obtained. This would be expected for the permeation of simple picrate anions. McLaughlin [9], using the same procedure, found a square relationship between λ_0 and the dinitrophenol concentration in water, consistent with the hypothesis of a HA_2^- complex as permeant species. Such complexes, however, may only be formed at pH values not too far from the pK of the weak acid HA. Since the pK value of dinitrophenol (about 4) is considerably larger than that of trinitrophenol (0.4), the different behaviour of both compounds appears understandable.

DISCUSSION

The experimental results which have been presented provide evidence for a facilitation of the transport of certain nitrophenolates induced by membrane-active macrocyclic compounds such as valinomycin. This evidence is mainly based on the following findings in potassium-free systems: (a) The increase in membrane conductance does not depend on the nature and valency of the cation. (b) The polarity and the magnitude of the diffusion potential in the presence of a concentration gradient of trinitrophenol implies that the picrate anion is the charge carrying species.

If K^+ is present in the aqueous phases, the conductance of the membrane is considerably larger compared to the potassium-free system. This behaviour mirrors the known cation specificity of valinomycin and supports the assumption that the conductance of the potassium system is mainly generated by the movement of positively charged valinomycin/K^+ complexes, while the transport of picrate anions in the concentration range below 10^{-3} M plays a minor role. On the other hand picrate anions seem to enhance the potassium transport by adsorbing to the membrane-water interface and by generating a negative surface potential (see also ref. 7). This interpretation is supported by the following results: (a) If the interfacial picrate concentration is corrected for the negative surface potential, a linear relationship between conductance and picrate concentration is obtained (Fig. 7). This is expected for a hydrophobic ion. (b) An increase of the ionic strength, which reduces the influence of the surface charges, leads to increase of the conductance (Fig. 5). The assumption of different conductance mechanisms for the potassium and the "potassium-free" systems provides a new explanation for the finding of Tosteson [8] of an apparent reduced K^+/Na^+ selectivity of valinomycin in the presence of trinitrocresolate anions.

The surprising result of an enhanced permeability of various nitrophenolate anions in the presence of macrocyclic compounds demands an attempt for a molecular interpretation. All three substances shown in Table II are known as carriers for cations, such as potassium. Therefore, one might imagine that they also act as carriers for certain anions. This would require the existence of stable carrier-anion complexes. The structure of these complexes must, however, be completely different from the structure of the cation complexes, since the size of a picrate anion is far too large to allow its incorporation into the interior of macrocyclic carriers (as in case of cations). Tosteson [8] and Davis and Tosteson [16] provided spectroscopic evidence for the existence of neutral ion pairs, including valinomycin, Na^+ and trinitrocresolate anions in homogeneous media. Such neutral complexes, however, do not contribute to charge transfer across membranes. Besides, a clear dependence on the cation concentration and also on the kind of cation would be expected, neither

of which were found. Nevertheless, a simple carrier mechanism for anions cannot be excluded on the basis of the experiments presented. But the question arises whether specific carrier-anion complexes must be postulated in order to explain facilitated transport. We will start by considering the non-facilitated transport of nitrophenolate-anions. The first step in their movement across a lipid membrane is their adsorption to an interface. Evidence has been presented that this adsorption generates a negative surface potential to the interface. Therefore, energy minima at the interfaces must exist, which are separated by an energy barrier representing the interior of the membrane. This relatively simple picture has been found to predict sufficiently well the transport of hydrophobic ions across lipid membranes [18–20]. The height of the energy barrier which limits the diffusion across the membrane is determined by several factors. Firstly, a specific interaction of the ion with the interface will enlarge the depth of the energy minima. Secondly, image forces acting on an ion near the interface of two media with different dielectric constants give rise to a broad barrier in the middle of the membrane [21]. Finally, the intermolecular interaction of adjacent hydrocarbon chains of the lipid molecules hinders the penetration of "foreign molecules". This interaction is fundamental for the maintainance of a liquid crystalline order inside the membrane. The diffusion of any solute is limited by the amount of free volume available in the frame of this structural order. Such free volume is present through the existence of defect structures. The diffusion of solutes across the hydrocarbon-like interior of biological and model membranes has been described in a way analogous to diffusion processes in polymers [22, 23]. Special defect structures found to be present in paraffins, in polyethylene and in other polymers [24], have also been suggested for lipid membranes and have been considered as "intrinsic carriers" for small solutes such as water molecules [25]. Larger molecules, however, do not fit into these intrinsic defect structures with a diameter of one to several Å in the lateral direction. The presence of valinomycin or nonactin (diameter 12–16 Å) must considerably disturb the order of their lipid environment, i.e. introduce additional disorder. Since the flexibility of hydrocarbon chains in a bilayer is limited the distortions in the proximity of those molecules will inevitably create additional free volume. This may be occupied by other, smaller "foreign molecules". In the light of these considerations the following possibility of facilitated diffusion may be envisaged (Fig. 8): A large "mediator M" allows the incorporation of a small solute

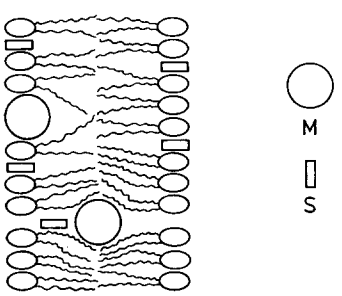

Fig. 8. Schematic representation of the transport of a species S facilitated by a mobile mediator M. The small molecule S can exist inside the free space generated by the large molecule M within the liquid crystalline lattice of lipid molecules. It moves concomitantly with M across the membrane.

S, otherwise largely confined to the interface, into the interior of the membrane by the generation of free volume in the environment of M. If M itself is mobile inside the membrane, it will transport "free volume" from one interface to the other and simultaneously solute S inside this volume. Though no direct chemical interaction between M and S is assumed (as in case of a carrier mechanism), a charged solute S will be able to transfer the driving force, exerted by an electric field, to the coupled movement of S and M.

The energy profile for the macrocyclic compounds valinomycin and nonactin has been found to be similar to that of hydrophobic ions by different methods [26, 27], They are preferentially confined to the membrane-water interface, but have a relatively high probability per unit time of crossing the barrier of the membrane interior. A kinetic analysis performed on the basis of voltage jump-current relaxation experiments yielded values for the translocation rate constant of the neutral species of about 10^3–10^5 s^{-1}, depending on the type of carrier and the kind of lipid [10, 11, 28–30]. A similar analysis of the hydrophobic ions dipicrylamine and tetraphenylboron gave values of 380 and 9 s^{-1}, respectively, for dioleoyllecithin membranes [18]. The high translocation rate constants of valinomycin and nonactin, in combination with their size, indicate that they might serve as mediators for the transport of picrate anions as illustrated in Fig. 8. With 10^{-7} M valinomycin in the aqueous phases about 10^{12} molecules are adsorbed to 1 cm^2 of the membrane composed of about $2 \cdot 10^{14}$ dioleoyllecithin molecules/cm^2 [11]. The application of the Gouy-Chapman theory of charged interfaces allows the calculation of the number of charges, which are responsible for a given electrical surface potential. From the data of Fig. 7 one calculates an adsorption density of about $3 \cdot 10^{13}$ picrate anions/cm^2 at 10^{-3} M picrate in the aqueous phases*. Though this number is only an approximation (see footnote), it might indicate a relatively dense packing of picrate anions at the membrane interface. This could considerably favor the occupation of free defect structures in the proximity of valinomycin molecules. Fig. 7 contains the "true" concentration dependence of the conductance for the pure picrate system (corrected for the negative surface potential). The same procedure carried out for the system valinomycin/picrate (data of Fig. 1) showed that the conductance depends roughly on the third power of the "actual" picrate concentration (not shown in Fig. 7). This seems to imply, in the light of the present argument, that the probability of occupation of empty defect structures generated by valinomycin is drastically enhanced with increasing packing density of picrate at the membrane surface. In other words, picrate anions prefer the adsorption sites at the interface at low packing density.

We will now deal with the time dependence of the conductance observed in the presence of picrate and valinomycin (or nonactin) (see Fig. 2). The slow decrease of the current following the application of a fixed voltage, ranging from milliseconds to seconds, is suggestive of diffusion polarization. This phenomenon is observed, if the voltage-dependent charge transport through the membrane is faster compared to the diffusion of these charge carriers through the unstirred layers adjacent to the

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* This calculation is only correct, if the change of the electrostatic potential "sensed" by nonactin/K^+ is essentially due to the negative charge and not due to a dipole moment of picrate. In addition, the applied procedure for the determination of $\Delta\Psi_0$ requires that other membrane parameters, such as the microviscosity, are not influenced by the adsorption of picrate [34].

membrane. It arises from a time-dependent increase and decrease of the charge carrier concentration at opposite membrane interfaces and has also been repeatedly observed in case of hydrophobic ions [18, 32, 2]. Nevertheless, the following arguments speak against the unstirred layers as the origin of this effect. The question whether diffusion polarization will be observed or not, depends on the ratio λ_{00}/c of initial conductance λ_{00} over the bulk concentration c of the permeating charge carriers. For $\lambda_{00}/c \ll 0.5$ $\Omega^{-1} \cdot cm^{-2} \cdot M^{-1}$, diffusion polarization at lipid membranes can be neglected [32]. From the data presented in Fig. 1 one calculates for the system nonactin/picrate at 10^{-2} M (assuming picrate as charge carrier): $\lambda_{00}/c = 0.01\ \Omega^{-1} \cdot cm^{-2} \cdot M^{-1}$. This number shows that the observed relaxation cannot be caused by unstirred layer effects, if picrate is the charge carrier. An even stronger argument comes from a comparison between the effects of valinomycin and nonactin. The amplitude of the current decrease has been found to be considerably larger for the system nonactin/picrate compared to valinomycin/picrate. This is in contrast to conclusions drawn from the amplitude of diffusion polarization, since the conductance in the presence of valinomycin exceeds that in the presence of nonactin (i.e. $(\lambda_{00}/c)_{\text{valinomycin/picrate}} > (\lambda_{00}/c)_{\text{nonactin/picrate}}$). Finally, the exact shape of the time-dependent current (Fig. 2), though extending over the same time range, does not agree with the time dependence of diffusion polarization measured for other systems (Stark, G., unpublished).

These arguments indicate that processes inside the membrane (i.e. not within the unstirred layers) seem to be responsible for the time dependence of the current in the presence of trinitrophenol and nonactin. The decrease of the current extends over at least four orders of magnitude in time and requires the assumption of at least four different time constants, if formally described as a series of exponentials. This reveals a rather complex mechanistic basis and in some way is reminiscent of non-specific structural effects. Within the frame work of the mechanism presented in Fig. 8 one might imagine that the desorption of picrate anions out of some of the sites produced by valinomycin or nonactin is hindered. This could lead to an accumulation of picrate anions on the positive side of the membrane and would produce a voltage difference across the membrane opposite to the applied one. If a large variety of structurally different "transport sites" exists, a series of different time constants will be present. Thus, the more or less continuous decrease of the current, also observed for the potassium system (i.e. in the presence of positive charge carriers in the membrane), would be attributed to the development of an asymmetry in the charge distribution on both sides of the membrane, i.e. the generation of an electrical potential difference. If the external voltage is removed, this asymmetry potential should persist over a period of time comparable to the time which was necessary for its development. This was confirmed by the experiment.

According to this interpretation, the initial conductance λ_{00} refers to the conductance of the symmetrical membrane, in which we are primarily interested. The quantitative differences for valinomycin and nonactin can consequently be explained on the basis of a small difference in the "defect structures" generated by these two kinds of mediators.

FINAL REMARKS

The experiments presented in this article have been interpreted on the basis of

a model for facilitated transport, which does not depend on the assumption of stable chemical bonds between a "transport mediator" and the transported substrate. Although the existence of a "normal" carrier mechanism (i.e. existence of stable bonds) for valinomycin-mediated picrate transport cannot be excluded at present, there is no necessity to postulate a direct interaction between mediator and substrate. Further experiments are necessary, however, to confirm the ideas outlined above. According to the model represented in Fig. 8, all molecules of sufficient size and free mobility inside the membrane could act as mediators for suitable substrates. One future aim should therefore be to look for the existence of further mediators of picrate and to study the question, whether valinomycin is also able to facilitate the transport of other surface-active substrates of similar size to picrate. Recently, a valinomycin-induced enhancement of the transport of the dye 8-anilino-1-napthalene-sulfonate across liposomes has been reported [33]. On the other hand, surface-active substances such as the uncoupler 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole and the hydrophobic ion tetraphenylborate have been found to block the valinomycin/K^+ system [6, 7], while picrate raises its effect. Which kind of interaction between, different transport systems will be observed, seems to depend on the precise nature of the molecules involved as well as on the structural properties of the membrane.

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